Neuron, Vol. 36, 213–228, October 10, 2002, Copyright ©2002 by Cell Press

Experimental Genetic Approaches to Addiction

Review

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Drugs of abuse are able to elicit compulsive drug-seeking behaviors upon repeated administration, which ultimately leads to the phenomenon of addiction. Evidence indicates that the susceptibility to develop addiction is influenced by sources of reinforcement, variable neuroadaptive mechanisms, and neurochemical changes that together lead to altered homeostasis of the brain reward system. Addiction is hypothesized to be a cycle of progressive dysregulation of the brain reward system that results in the compulsive use and loss of control over drug taking and the initiation of behaviors associated with drug seeking (Koob et al., 1998). The view that addiction represents a pathological state of reward provides an approach to identifying the factors that contribute to vulnerability, addiction, and relapse in genetic animal models.

Most of the advancements in our understanding of neural processes leading to addiction come from animal studies. Any addictive drug of abuse works as a positive reinforcer in virtually all vertebrate species. The fact that the pathological endpoint can be achieved by pharmacological means in experimental animals makes addiction easier to study than most of the other neuropsychiatric disorders. Still, the identification of the genes and proteins whose orchestrated function is responsible for complex behaviors is a formidable task, and drug-taking behavior represents one of the most complex manifestations of central nervous system (CNS) dysfunction. In this review, we examine how experimental genetic approaches have been utilized to resolve the mechanisms of action of different drugs of abuse and the plastic neuronal changes that underlie addiction. For more general overviews of the neurobiology of addiction, we direct the reader to several excellent recent reviews (Wise, 1996; Nestler and Aghajanian, 1997; White and Kalivas, 1998; Koob et al., 1998; Di Chiara et al., 1999; Berke and Hyman, 2000; Nestler, 2000, 2001a; Hyman and Malenka, 2001). Here, we concentrate on a few, more specific, topics: what are the most relevant behavioral paradigms for evaluating mutant animals? How have the candidate gene approach and development of knockout mouse strains contributed to our understanding of the subject? And, finally, how may the forward genetics approach help us to identify new drug targets and explore truly novel realms of addiction mechanisms? Most of the examples given are related to cocaine and other psychostimulants, but the basic principles of the research described can be applied to most drugs of abuse.

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Behavioral Manifestations of Drugs of Abuse

Addiction can be approached with the tools of genetic analysis due to the establishment of behavioral paradigms that model different aspects of the progression toward a pathological state. The endpoint of addiction involves a series of behavioral manifestations that are variably evident, depending on the particular drug of abuse (Woolverton and Johnson, 1992). All drugs of abuse produce an acute response, which is most often characterized by enhanced arousal or euphoria. In rodent studies, enhanced arousal is predominantly observed as increased locomotor activity and stereotypic behaviors. The ability to induce euphoria and reward is usually studied using self-administration and conditioned place-preference paradigms. In the self-administration test, the animal can be trained to control drug intake by nose poking (Kuzmin et al., 1992) or lever pressing (Caine et al., 1999), whereas conditioned place preference is based on the animal's ability to pair rewarding drug with environmental cues in a two-chamber apparatus (Woolverton and Johnson, 1992).

Tolerance refers to a situation where increasing doses of a drug become necessary to elicit an equivalent physiological response. This behavioral manifestation is usually best exemplified when studying the antinociceptive or rewarding effects of morphine (Di Chiara and North, 1992). Concurrent with tolerance and yet opposite in nature is the phenomenon of behavioral sensitization, whereby repeated intermittent exposure to a drug leads to a progressive enhancement of the response to that drug. Repeated administration of cocaine, for example, produces more pronounced locomotor stimulation upon each consecutive exposure in many animals (Post and Rose, 1976). The coexistence of tolerance and sensitization may first seem counterintuitive, but they probably reflect different aspects of drug action. For instance, continuous cocaine infusion may lead to tolerance, whereas intermittent exposure to the same drug leads to sensitization (Post and Rose, 1976; Inada et al., 1992; King et al., 1992; Izenwasser and French, 2002). While it is difficult to assess cocaine sensitization in humans, it is an established phenomenon in animals and represents an enduring alteration in drug response. Psychostimulant sensitization is discussed in more detail below.

Another behavioral manifestation is physical dependence, which corresponds to an adaptive state of the cells, circuits, or organ systems that are unmasked by abrupt cessation of drug exposure. This property is also most often associated with opiate treatment, as opioid receptor antagonists induce a state of withdrawal that is noticeably unpleasant for the animal (Di Chiara and North, 1992). A further behavioral consequence of drug abuse is drug craving, the extent of which can be studied by measuring drug-seeking behaviors (Littleton, 2000). This manifestation may be common to most drugs of abuse. Compulsive drug taking, in which uncontrolled drug self-administration continues despite noxious physical and social consequences, is an advanced manifestation of the addiction process. Lastly is the phenom-

enon of relapse; animals are trained to self-administer drug, the compulsive drug-taking behaviors are then extinguished, and there is an increased tendency following conditioned stimulus to reacquire the drug-taking behavior (Littleton, 2000; Shalev et al., 2002). Some have argued that this behavior may be a more, if not the only, relevant paradigm of drug addiction in humans. These different manifestations are likely to involve changes in different neurotransmitter systems and neural circuits in the brain. An outstanding question in the field of drug abuse is whether all of these behavioral manifestations are directly associated with the addictive process or are simply epiphenomena.

The study of addiction using genetically manipulated animals is still relatively young and has concentrated only on few of the aforementioned paradigms. Acute responses to psychostimulants or opiates have been studied in many mutant mice, but less is known about the genes contributing to tolerance, sensitization, and reward. Genetic mechanisms regulating more complex behaviors (such as relapse) are virtually unknown. In a few years from now, it should be possible to organize such a review along the groups of genes that affect each of these different behavioral manifestations. At present, however, we feel that the reader is still best served by categorizing the existing literature according to different levels of neurotransmitter signaling.

Neurotransmitters and Neural Circuits Involved in Addiction: Identifying Candidate Genes

Although different classes of drugs of abuse have different molecular targets in the CNS, they all lead to increases in extracellular concentrations of the monoamine dopamine in certain regions in the brain. Dopamine is a neurotransmitter that is involved in the control of locomotion, cognition, emotion, affect, and reward (Carlsson, 1987). In addition to the dopaminergic system, serotonergic and noradrenergic systems contribute, for example, to the effects of psychostimulants. Moreover, the targets of other classes of abused drugs, such as opiates, alcohol, and nicotine, encompass other major neurotransmitter pathways, such as endogenous opioids, the GABAergic system, and the cholinergic system, respectively. The brain regions thought to be most involved in the reward, mood, cognition, and arousal processes important for addiction are the nucleus accumbens (particularly the shell), the prefrontal cortex, the ventral tegmental area (VTA)/substantia nigra region where dopamine cell bodies are located, and other brain regions referred to as extended amygdala (Koob et al., 1998; Di Chiara et al., 1999). It may become evident that neural circuits are more important for reward and compulsive drug intake rather than specific neurotransmitter systems. For example, the brain reward system that has been suggested to consist of extended amygdala is neurochemically a very heterogenous circuit. Moreover, the manifestation of the addicted state is hypothesized to involve brain circuits critical for any compulsive behavior, such as the cortico-striatal-thalamic loop (Volkow and Fowler, 2000; Porrino and Lyons, 2000; Koob and Le Moal, 2001; Hyman and Malenka, 2001). Consequently, one might expect that a large number of individual genes might contribute to the manifestations of the addictive state.

The search for potential candidate genes participating in the development of addiction can be illustrated by using the example of behavioral sensitization (Woolverton and Johnson, 1992; Wise, 1996; White and Kalivas, 1998; Nestler, 2001a). In general, repeated administration of psychostimulants results in the initiation and intensification of the many biochemical and behavioral manifestations that ultimately lead to the addicted state. Behavioral sensitization is thought to be one of the early manifestations of neuronal plasticity associated with chronic administration of a drug of abuse. The increased sensitivity is often generalized across many drugs of abuse; for example, sensitization to cocaine engenders an enhanced response to amphetamine and even to heroin. Sensitization to the locomotor stimulating effects of psychostimulants is relatively simple to document in animal models and is one of the most reproducible behavioral paradigms to study. Along with the acute response, it is also probably the best studied in the context of genetically manipulated animals. Sensitization induces long-lasting changes in the animal's response to a drug, enduring weeks and even months after the last dose. While the biochemical basis of this enhanced responsiveness is still not clear, sensitization is believed to be associated with augmented intensity of dopaminergic transmission (Pontieri et al., 1995; White and Kalivas, 1998). For example, both increased sensitivity of postsynaptic dopamine receptors (supersensitivity) and increased output of dopamine from presynaptic terminals have been postulated to contribute to sensitization (Woolverton and Johnson, 1992; White and Kalivas, 1998; Nestler, 2001a).

Although dopamine represents a major neurotransmitter system involved in addiction, it has become increasingly clear that other neurotransmitters can also significantly contribute to this pathological condition. For instance, psychostimulants are known to be potent blockers not only of dopamine transporter (DAT), but also of the other monoamine transporters, namely the norepinephrine (NET) and serotonin (SERT) transporters (Amara and Kuhar, 1993). Recent evidence in genetically engineered mice suggests that both the noradrenergic and serotonergic systems may, in addition to the dopaminergic system, play significant modulatory roles in determining the pharmacological and behavioral actions of psychostimulants (Gainetdinov et al., 2002a). Therefore, genes involved in the control of these neurotransmitter systems may also contribute to the neuronal plasticity following chronic psychostimulant exposure. Genes encoding autoreceptors governing release, synthesis, and firing rate (Roth and Elsworth, 1995); synthetic and degradative enzymes; vesicular and plasma membrane transporters; and presynaptic heteroreceptors (glutamate, GABA, adrenergic, and serotonin) regulating dopamine are all candidate genes that could contribute to changes in responsiveness. Similarly, postsynaptic receptors, G protein subtypes, kinases, effector proteins, cytoarchitectural proteins, and metabolic enzymes are also valid candidates for substrates of supersensitivity (Nestler and Aghajanian, 1997). Other candidate genes that could mediate this long-term plasticity include transcription factors of the Jun/Fos family (Nestler and Aghajanian, 1997; Nestler, 2001a; Nestler et al., 2001). Lastly, recent electrophysiological experi-

ments have demonstrated that exposure to cocaine or amphetamine induces long lasting neuronal plasticity in glutamate transmission in the corticostriatal pathway and VTA (Jones et al., 2000; Ungless et al., 2001; Thomas et al., 2001; Beurrier and Malenka, 2002). Given the plethora of possible mechanisms that could underlie even a process as simple as sensitization, it is virtually inconceivable that, using conventional approaches, one could arrive at determining all of the relevant changes associated with, or causative of, addiction.

Classical Genetic Approaches: Forward and Reverse Genetics

Most of the knowledge available today about the molecular events underlying the drug abuse process has come long before the era of modern genetic research, using classical pharmacological, biochemical, and behavioral approaches. Identification of the principal targets of drugs of abuse has prompted the use of an approach referred to as reverse genetics. Reverse genetics begins with a gene and then looks for the phenotype that results from mutations in that gene. Forward genetics proceeds from an altered phenotype and looks for the gene or genes responsible for that phenotype. In the context of addiction, forward genetics has been applied only to a few organisms that demonstrate an altered response to a drug of abuse. Below, we will highlight some of the most informative examples of each approach to demonstrate the power of genetic analysis to this field of biology.

Reverse Genetics: A Candidate Gene Approach

Many candidate genes have been studied by assessing the behavioral consequences of null mutations in mice. Often, this approach has confirmed the postulated involvement of drug targets. It has been used to demonstrate the importance of μ-opioid receptors for the actions of morphine (Matthes et al., 1996), DAT for cocaine and amphetamine (Giros et al., 1996; Jones et al., 1998), NMDA receptors for phencyclidine (PCP) (Mohn et al., 1999), cannabinoid CB_1 receptors for cannabinoids (Ledent et al., 1999), and nicotinic acetylcholine receptors for nicotine (Picciotto et al., 1998). Sometimes exploration beyond the obvious has produced interesting results, indicating, for instance, that dopamine D2 receptors (Maldonado et al., 1997; Elmer et al., 2002) and substance P receptors (Murtra et al., 2000) are also involved in the rewarding effects of morphine. Likewise, the number of mutant mice known to display altered responses to the psychostimulants cocaine and amphetamine is already surprisingly large, especially considering the relatively simple pharmacology of these drugs (Table 1).

Monoamine Transporters as Targets of Psychostimulants

Sometimes it is necessary to knock out several candidate genes one by one and to try to narrow down the potential list of the principal drug targets. Cocaine, amphetamine, and many other psychostimulants are believed to produce their stimulating and addictive effects mainly by increasing the extracellular levels of dopamine in the brain reward areas through the interaction with DAT (Amara and Kuhar, 1993). Indeed, mice lacking the DAT are hyperactive, resembling animals treated chronically with cocaine, and psychostimulants do not increase further their locomotor activity (Giros et al., 1996; Gainetdinov et al., 1999). However, these mice still self-administer cocaine, as well as show conditioned place preference for cocaine, indicating that the drug's rewarding effects are not abolished (Rocha et al., 1998a; Sora et al., 1998). Could this mean that DAT is not important for cocaine's actions after all? The most likely answer is that it is still the most important mediator of cocaine's effects in wild-type animals, as well as in human abusers (Amara and Kuhar, 1993). However, since cocaine also inhibits the uptake of other monoamines, these findings led to a proposal that its ability to elevate synaptic serotonin or norepinephrine levels might contribute to its reinforcing effects. Although selective serotonin (SERT) and norepinephrine (NET) inhibitors (such as clinically used antidepressants) possess very little, if any, abuse potential in normal subjects, the lack of cocaine's primary target in DAT knockout mice might have brought forth this subtle component of cocaine's action. Moreover, one has to remember that the sustained genetic blockade of DAT leads to a situation where cocaine-induced increase in the extracellular levels of other monoamines is always coupled to a simultaneous (albeit chronic) elevation of extracellular dopamine levels–basically mimicking the effect of cocaine in wild-type animals. In other words, these studies do not exclude the possibility that elevated dopamine levels in the striatum and nucleus accumbens are still prerequisite for the rewarding effects of cocaine, even if serotonin and norepinephrine also have roles.

Studies in other monoamine transporter knockouts, however, suggest a more complicated interplay of these transmitters. Mice lacking either SERT or NET display enhanced cocaine place preference (Sora et al., 1998; Xu et al., 2000a), and this behavior is even further accentuated in mice lacking both transporters (Hall et al., 2002). This result may indicate that genetic lack of SERT and/or NET leads to a cross-sensitized cocaine response through the same mechanism as chronic antidepressant treatment (Spyraki and Fibiger, 1981; Rossetti et al., 1991; Xu et al., 2000a). It has also been suggested that serotonin and norepinephrine may mediate aversive effects of cocaine. Elimination of these components in NET/SERT knockout mice would leave only cocaine's "pure" dopamine-mediated reward (Sora et al., 2001; Hall et al., 2002). These explanations seem reasonable but leave the question of why, then, do DAT knockout mice display clear conditioned place preference not only for cocaine, but also for fluoxetine, a selective SERT inhibitor, and nisoxetine, a selective NET inhibitor (Hall et al., 2002)?

Two unexpected characteristics of DAT knockout mice might provide insight to this apparent paradox. First, both cocaine and fluoxetine actually lead to a dramatic calming effect in DAT knockout mice instead of increasing the already very high basal behavioral activity of these animals, and this effect has been shown to be mediated by the activation of the brain serotonergic system (Gainetdinov et al., 1999). Maybe these animals, suffering from chronic hyperactivity and restlessness, find SERT blockade caused by these drugs pleasant

Table 1. Knockout and Transgenic Mouse Strains Showing Altered Behavioral Responses to Psychostimulants (Cocaine and Amphetamine)

Strain	Reference	Acute Locomotor Response	Sensitization	Conditioned Place Preference	Self-Administration
Monoamine transporters					
DAT$^{-/-}$	Giros et al., 1996 Rocha et al., 1998a Sora et al., 1998 Gainetdinov et al., 1999	Stimulants inhibit activity	N/A	Present (C)	Delayed but present (C)
SERT$^{-/-}$	Sora et al., 1998, 2001 Bengel et al., 1998	Present	N/A	Enhanced (C)	N/A
NET$^{-/-}$	Xu et al., 2000a	Supersensitive	Do not sensitize further (C)	Enhanced (C)	N/A
DAT/SERT$^{-/-}$	Sora et al., 2001	No stimulatory effect (C)	N/A	Absent (C)	N/A
NET/SERT$^{-/-}$	Hall et al., 2001	N/A	N/A	Enhanced (C)	N/A
VMAT2$^{+/-}$	Wang et al., 1997 Takahashi et al., 1997	Supersensitive	Do not sensitize further (C)	Attenuated (A)	N/A
Dopamine receptors					
D1$^{-/-}$	Xu et al., 1994, 2000b Miner et al., 1995 Karper et al., 2002	Significantly attenuated (stimulants may inhibit activity)	Attenuated (C), intact (A)	Intact (C)	N/A
D2$^{-/-}$	Caine et al., 2002 Chausmer et al., 2002	Slightly attenuated (C)	N/A	N/A	Intact or slightly enhanced (C)
D3$^{-/-}$	Xu et al., 1997 Carta et al., 2000	Supersensitive (C)	N/A	Enhanced (A)	N/A
D4$^{-/-}$	Rubinstein et al., 1997	Supersensitive (C)	N/A	N/A	N/A
Glutamate receptors					
mGluR1$^{-/-}$	Mao et al., 2001	Supersensitive (A)	N/A	N/A	N/A
mGluR5$^{-/-}$	Chiamulera et al., 2001	Absent (C)	N/A	N/A	Absent (C)
GluR1$^{-/-}$	Vekosvischeva et al., 2001	N/A	Enhanced (A)	N/A	N/A
NR1$^{-/-}$ (hypomorph)	Laakso et al., 2001, Soc. Neurosci. Abstr.	Significantly attenuated (C), intact (A)	Intact (C)	Delayed but present (C)	N/A
Miscellaneous G protein-coupled receptors					
5-HT$_{1B}^{-/-}$	Rocha et al., 1998b	Supersensitive (C)	Intact (C)	N/A	Enhanced (C)
α1b$^{-/-}$	Drouin et al., 2002	Significantly attenuated	Attenuated	N/A	N/A
α2c$^{-/-}$	Sallinen et al., 1998	Supersensitive (A)	N/A	N/A	N/A
α2c overexpressing	Sallinen et al., 1998	Attenuated (A)	N/A	N/A	N/A
M1$^{-/-}$	Gerber et al., 2001	Supersensitive (A)	N/A	N/A	N/A
CCK$_2^{-/-}$	Kõks et al., 2001	Attenuated at low doses, supersensitive at high doses (A)	N/A	N/A	N/A
A$_{2A}^{-/-}$	Chen et al., 2000	Attenuated	N/A	N/A	N/A
G protein-coupled receptor signaling					
GRK6$^{-/-}$	Gainetdinov et al., 2002b	Supersensitive	Significantly attenuated (C)	N/A	N/A
G$_{OLF}\alpha^{-/-}$	Zhuang et al., 2000	Absent (C)	N/A	N/A	N/A
G$_z\alpha^{-/-}$	Yang et al., 2000	Supersensitive (C)	N/A	N/A	N/A

(*continued*)

Table 1. Continued

Strain	Reference	Acute Locomotor Response	Sensitization	Conditioned Place Preference	Self-Administration
G protein-coupled receptor signaling					
PDE1B$^{-/-}$	Reed et al., 2002	Supersensitive (methamphetamine)	N/A	N/A	N/A
PKA-RIIβ$^{-/-}$	Brandon et al., 1998	Intact	Enhanced (A)	N/A	N/A
Miscellaneous ion channel receptors					
GABA$_A$-β$_3^{-/-}$	Resnick et al., 1999	Supersensitive (C), intact (A)	Do not sensitize further (C)	N/A	N/A
nACHR-β$_2^{-/-}$	Zachariou et al., 2001	N/A	N/A	Attenuated at low doses (C)	N/A
Neurotrophic factors					
BDNF$^{+/-}$	Horger et al., 1999	Attenuated (C)	Slightly delayed	N/A	N/A
GDNF$^{+/-}$	Messer et al., 2000	Intact (C)	Enhanced (C)	Enhanced at low doses (C)	N/A
Transcription factors					
RARβ/RXRβ$^{-/-}$ RARβ/RXRγ$^{-/-}$ RXRβ/RXRγ$^{-/-}$	Krezel et al., 1998	Significantly attenuated (C)	N/A	N/A	N/A
*Fos*B$^{-/-}$	Hiroi et al., 1997	Supersensitive (C)	Do not sensitize further (C)	Enhanced at low doses (C)	N/A
ΔfosB overexpressing	Kelz et al., 1999	Supersensitive (C)	Intact (C)	Enhanced at low doses (C)	N/A
Miscellaneous					
mPer1$^{-/-}$	Abarca et al., 2002	Intact (C)	Absent (C)	Absent (C)	N/A
mPer2$^{-/-}$	Abarca et al., 2002	Intact (C)	Enhanced (C)	Intact (C)	N/A
DARPP-32$^{-/-}$	Fienberg et al., 1998 Zachariou et al., 2002	Intact (C)	Enhanced (C)	Attenuated (C)	N/A
Inhibitor 1$^{-/-}$	Zachariou et al., 2002	Intact (C)	N/A	Attenuated (C)	N/A
GR$^{-/-}$ (antisense)	Steckler and Holsboer, 1999	Supersensitive (A)	N/A	N/A	N/A
tPA$^{-/-}$	Ripley et al., 1999	Supersensitive (C)	Enhanced (C)	N/A	Intact

Abbreviations: C, cocaine; A, amphetamine; +/−, heterozygous for null mutation.
References for monoamine transporters: Giros et al., 1996; Rocha et al., 1998a; Gainetdinov et al., 1999; Sora et al., 1998, 2001; Bengel et al., 1998; Xu et al., 2000a; Hall et al., 2001; Wang et al., 1997; Takahashi et al., 1997.
References for dopamine receptors: Xu et al., 1994, 2000b; Miner et al., 1995; Karper et al., 2002; Caine et al., 2002; Chausmer et al., 2002; Xu et al., 1997; Carta et al., 2000; Rubinstein et al., 1997.
References for glutamate receptors: Mao et al., 2001; Chiamulera et al., 2001; Vekosvischeva et al., 2001; Laakso et al., 2001, Soc. Neurosci. Abstr.
References for miscellaneous G protein-coupled receptors: Rocha et al., 1998b; Drouin et al., 2002; Sallinen et al., 1998; Gerber et al., 2001; Kõks et al., 2001; Chen et al., 2000.
References for G protein-coupled receptor signaling: Gainetdinov et al., 2002b; Zhuang et al., 2000; Yang et al., 2000; Reed et al., 2002; Brandon et al., 1998.
References for miscellaneous ion channel receptors: Resnick et al., 1998; Zachariou et al., 2001.
References for neurotrophic factors: Horger et al., 1999; Messer et al., 2000.
References for transcription factors: Krezel et al., 1998; Hiroi et al., 1997; Kelz et al., 1999.
References for miscellaneous: Abarca et al., 2002; Fienberg et al., 1998; Zachariou et al., 2002; Steckler and Holsboer, 1999; Ripley et al., 1999.

because of its soothing effect instead of experiencing further reward in a classical sense? This explanation would not be in contradiction with somewhat aversive effects of acute SERT blockade in normal subjects. In line with this hypothesis, mice lacking both DAT and SERT no longer display cocaine place preference (Sora et al., 2001).

Cocaine, amphetamine, and the selective NET inhibitor, reboxetine, were shown to increase extracellular dopamine levels selectively in the nucleus accumbens, but not in the dorsal striatum, of DAT knockout mice (Carboni et al., 2001). This potentially explained why cocaine and nisoxetine were still rewarding in these mice and was proposed to reflect blockade of NET-mediated dopamine uptake into noradrenergic nerve terminals. Heterologous uptake of dopamine via the NET in brain areas with low DAT levels has been, in fact, recently documented (Moron et al., 2002). More detailed investigation, however, ruled out any direct effects of cocaine or selective NET inhibitors on dopamine clearance in the nucleus accumbens in DAT knockout animals (Budygin et al., 2002). Instead, cocaine and NET inhibitors seem to act outside the nucleus accumbens to increase dopamine release in mesolimbic dopaminergic neurons in DAT knockout mice. One possible circuit mediating the effects of norepinephrine on mesolimbic dopamine release may involve α1-adrenoceptors (Darracq et al., 1998). This may be, at least in part, a reason why α1b-adrenoceptor knockout mice do not display cocaine-induced hyperactivity and reward (Drouin et al., 2002). However, a similar role of serotonin in the regulation of mesolimbic dopamine release cannot be excluded. These findings suggest a role for the mesolimbic dopamine system in the reinforcing effects of cocaine and other psychostimulants, even in DAT knockout mice. One caveat, however, is that it is unclear whether a further increase in already dramatically elevated dopamine levels can actually be rewarding.

The brain vesicular monoamine transporter (VMAT2) pumps monoamines, including dopamine, from the cytoplasm into storage vesicles from which neurotransmitters are released into the synaptic cleft upon neural impulse. Homozygous VMAT2 null mutant mice completely lack the ability to store catecholamines in the CNS and die soon after birth (Wang et al., 1997; Takahashi et al., 1997; Fon et al., 1997). However, heterozygote animals are viable and show a 25% decrease in the tissue content and 40% decrease in the basal extracellular levels of dopamine, resembling reserpine-treated animals (Wang et al., 1997). This chronic deficiency of the endogenous agonist leads to a denervation-type supersensitivity of postsynaptic dopamine receptors. Accordingly, the acute locomotor responses to psychostimulants in heterozygous mice are increased to a level that is comparable to maximally sensitized, wild-type animals despite significantly attenuated stimulated dopamine release (Wang et al., 1997). However, these animals still show reduced amphetamine-conditioned place preference (Takahashi et al., 1997), suggesting that locomotor-stimulating and -rewarding properties of a reinforcing drug are not necessarily always correlated.

Overall, studies in monoamine transporter knockout animals paint a picture of considerable complexity in the actions of cocaine (Gainetdinov et al., 2002a). The most surprising observation is the preserved reward in dopamine transporter knockout mice. While these data suggest that serotonergic and noradrenergic components are important to both the reinforcing and aversive effects of cocaine, they also demonstrate what powerful tools genetically manipulated animals can be. This level of dissection of the pharmacological actions of cocaine had not been achieved prior to the availability of knockout animals.

Dopamine Receptors Mediating Reward

Studies in various dopamine receptor knockout mice have also strongly implicated the dopamine system in drug abuse. D1 receptor is the most abundant dopamine receptor in the brain, outnumbering the D2 receptor by 3-fold in the striatum. It was also the first dopamine receptor to be genetically altered in the mouse (Xu et al., 1994). As expected, cocaine did not cause hyperactivity in D1 receptor knockout mice and also lost its ability to induce the expression of various early genes, such as c-*fos* and *junB* in the striatum of knockout mice (Moratalla et al., 1996). Stimulant-induced gene expression is thought to participate in the plasticity underlying long-term effects of drugs of abuse (Nestler et al., 2001; Nestler, 2001a), and D1 mutant mice indeed display reduced sensitizing response to repeated cocaine administration (Xu et al., 2000b). Quite surprisingly, though, cocaine-induced reward assessed by conditioned place preference is intact in D1 receptor knockout mice, suggesting that signaling through other dopamine receptors is sufficient to preserve the reward mechanisms (Miner et al., 1995). Mice lacking the other member of the D1-like receptor subfamily, the D5 dopamine receptor, have normal basal activity and display somewhat decreased response to D1-like receptor agonist SKF-81297 (Holmes et al., 2001), but their response to psychostimulants has not been yet reported.

The consequences of D2 receptor deletion may seem even more puzzling. D2 receptor knockout mice do self-administer cocaine, can identify cocaine in a drug discrimination test, and increase their locomotor activity in response to cocaine (Caine et al., 2002; Chausmer et al., 2002). Behavioral response in conditioned place preference for psychostimulants has not been reported. However, they fail to show conditioned place preference to morphine (Maldonado et al., 1997) or to self-administer this drug (Elmer et al., 2002), the effects of which are presumably mediated mostly by μ-opioid receptors (Matthes et al., 1996). They also have lower propensity to self-administer ethanol (Risinger et al., 2000) and respond at a lower rate than wild-type mice to food and water reinforcers during training periods (Caine et al., 2002; Elmer et al., 2002). This suggests that while D2 receptor function is generally important for many natural and exogenous rewards (Kelley and Berridge, 2002), the high potency of cocaine to act as a reinforcer may again overcome this defect through other signaling pathways.

Mice lacking the other two members of the D2-like receptor subfamily, the D3 and D4 dopamine receptors, also show interesting phenotypes. Like D2 receptors, D3 receptors are expressed both as pre- and postsynaptic dopamine receptors (Diaz et al., 2000). D3 receptor knockout mice are slightly hyperactive in a novel envi-

ronment, supersensitive to the locomotor effects of cocaine, and display increased reward response to amphetamine as assessed by conditioned cue-preference paradigm (Xu et al., 1997). In addition, basal extracellular levels of dopamine are higher in mutant mice, which may provide explanation to their heightened sensitivity to psychostimulants (Koeltzow et al., 1998; Joseph et al., 2002). D4 dopamine receptor knockout mice are hypoactive in a novel environment but show supersensitive locomotor responses to ethanol, cocaine, and methamphetamine (Rubinstein et al., 1997).

Clearly, more studies are needed to investigate the contribution of these receptors to the rewarding effects of drugs of abuse. It is also worth noting that none of the dopamine receptors have been found to be essential for the rewarding effects of cocaine. Importantly, this preservation of reward does not seem to be due to compensatory upregulation of remaining dopamine receptor subtypes; D1 knockout mice show a minor (20%) decrease in striatal D2 receptor binding, and deletions of D2, D3, or D4 seem to be devoid of any notable compensatory changes in other dopamine receptors (Xu et al., 1994, 1997; Rubinstein et al., 1997; Kelly et al., 1998). Instead, these findings indicate either the existence of redundancy in dopaminergic reward pathways or the importance of other monoamines when dopaminergic signaling is not intact. These hypotheses could be tested using double or multiple knockouts, as has been done in the case of monoamine transporter knockouts. The data may also suggest that dopamine receptor antagonists may not be promising targets for pharmacotherapy of cocaine addiction. On the other hand, partial agonists of various dopamine receptors have shown some promise by counteracting the rapid elevations in extracellular dopamine concentrations induced by psychostimulants and by having low abuse potential themselves while still providing "substitution" to stimulant-induced receptor activation (Pulvirenti and Koob, 2002).

Intracellular Signaling Mutants and Addictive Drugs

Traditional addiction research has focused largely on release of endogenous neurotransmitters and the immediate effect they, or the drugs mimicking them, have on their membrane-bound receptors. However, especially for the long-term effects of drugs of abuse, what happens inside the cell is probably more important than what happens between the brain cells (Nestler and Aghajanian, 1997; Koob et al., 1998; Robbins and Everitt, 1999; Berke and Hyman, 2000; Hyman and Malenka, 2001; Nestler, 2001a). Stimulation of neurotransmitter receptors leads to signaling cascades involving G proteins and their effectors, second messengers, protein kinases and phosphatases, modification of intracellular proteins, activation of transcription factors, and long-lasting changes in the gene expression. When targeted in the right cell and right region of the brain, any single component in this chain of events is likely to modify either acute responses to drugs of abuse or the persistent changes they are capable of inducing. Candidate genes for intracellular proteins that modulate drug reward can be sorted roughly into two classes: those that mediate the initial response, such as receptor and second messenger activation, and those that participate in long-term changes in neuronal plasticity (Figures 1 and 2).

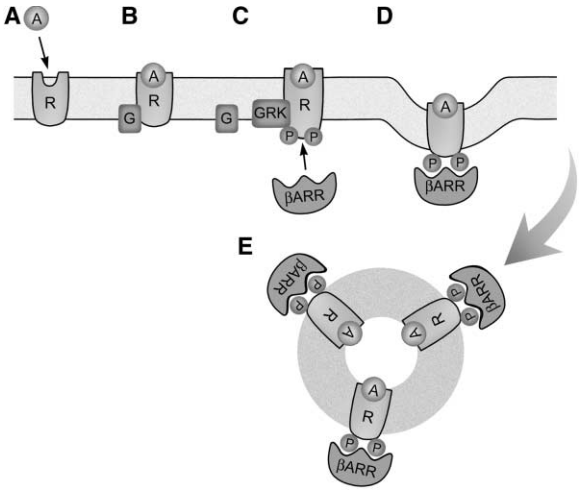

Figure 1. Desensitization Mechanisms of G Protein-Coupled Receptors Can Affect the Efficacy of Many Drugs of Abuse

(A and B) Binding of the agonist (A) to the receptor (R), such as those for dopamine or opioids, leads to coupling of the receptor to the G protein (G) and activation of the latter.

(C–E) In (C), G protein-coupled receptor kinases (GRK) can phosphorylate (P) receptors, either upon agonist stimulation or sometimes under basal conditions. This leads to the binding of β arrestins (βARR) and other accessory proteins to the receptor, uncoupling from G proteins, and finally to (D and E) internalization and either recycling or degradation of the receptor (Claing et al., 2002; Pierce and Lefkowitz, 2001).

G protein-coupled receptor kinases (GRKs) phosphorylate G protein-coupled receptors, leading to the binding of β arrestins and other accessory proteins to the receptor; the uncoupling of the receptor from G proteins; and, finally, endocytosis; thus promoting desensitization of receptor signaling (Claing et al., 2002; Pierce and Lefkowitz, 2001). Genetic deletion of GRKs therefore presumably leads to supersensitive G protein-coupled receptors. Which receptors will be the ones affected depends on the colocalization with a given GRK subtype (five of which, GRK2–GRK6, are found in the brain) to the same neurons and brain regions, as well as the possibility of biochemical interaction between the kinase and the receptor. As an example, GRK6 is widely expressed in the brain, including striatum and cortical areas, and mice lacking GRK6 are supersensitive to cocaine and amphetamine (Gainetdinov et al., 2002b). Dopamine release in these mice is unaffected, and the supersensitivity to stimulants is most likely explained by increased sensitivity of postsynaptic D2-like dopamine receptors. These findings may open new avenues for the etiological research and pharmacological treatment of disorders associated with aberrant dopaminergic neurotransmission.

G proteins mediate the intracellular responses to the stimulation of G protein-coupled receptors. G proteins consist of three subunits, α, β, and γ. The specificity of receptor G protein interaction, as well as the appropriate second messenger effector, is determined by the G_{α} subunit. The $G_{OLF\alpha}$ subunit, named after its prominent

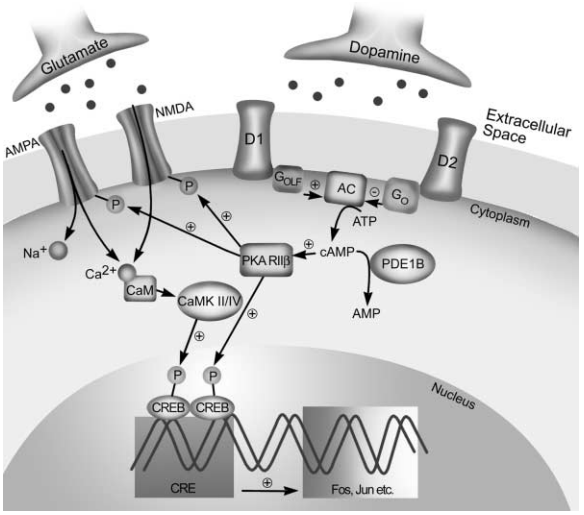

Figure 2. Interactions of Different Neurotransmitters in the Nucleus Accumbens Are Mostly Responsible for the Acute Effects as Well as Long-Term Neuronal Plasticity that Contribute to the Development of Addiction

Medium spiny neurons in striatal brain regions receive, e.g., nigrostriatal and mesolimbic dopaminergic innervation and corticostriatal glutamatergic innervation. D1-like (D1) and D2-like (D2) dopamine receptors stimulate and inhibit adenylyl cyclase (AC), respectively, through specific G_α subunits of G proteins (G_{OLF} and G_O). Cyclic AMP (cAMP) formed by AC activates cAMP-dependent protein kinases (e.g., PKA-RIIβ) and is itself inactivated by phosphodiesterases (e.g., PDE1B). PKA, in turn, regulates the ion conductivity of AMPA- and NMDA-type glutamate receptors by phosphorylating (P) different receptor subunits. Ionic influx (Na^+, Ca^{2+}) through glutamate receptors regulates the membrane potential of the neuron and activates Ca^{2+}/calmodulin-dependent kinases (CaMKII/IV) via calmodulin (CaM). Both CaMK and PKA regulate gene expression by influencing the phosphorylation state of different transcription factors, such as the cAMP-responsive element binding protein (CREB). Phosphorylated CREB activates the transcription of numerous genes having cAMP-responsive element (CRE) in their promoter regions, such as members of the Fos/Jun family of transcription factors (c-Fos, FosB, c-Jun etc.). Other known components relevant for these signaling pathways that are not shown include regulators of G protein signaling (RGS proteins), members of the MAP kinase pathway, phosphatase/kinase inhibitor DARPP-32, and several phosphatases that interact with it (e.g., PP-1 and calcineurin) (Nestler, 2001a; Greengard, 2001; Jiang et al., 2001; and other references within the text).

expression in the olfactory epithelium, also mediates the majority of D1 dopamine receptor-mediated adenylyl cyclase activation in the striatum (Zhuang et al., 2000). Like D1 receptor knockout mice, $G_{OLF\alpha}$ knockout mice are–paradoxically–slightly hyperactive but do not respond to acute cocaine administration by increasing their locomotor activity and also do not display induction of striatal c-Fos expression in response to cocaine. In contrast, mice lacking the $G_{z\alpha}$ subunit, which inhibits adenylyl cyclase, show increased locomotor response to acute cocaine administration (Yang et al., 2000). However, the receptor(s) mediating this effect in the brain is not known.

While repeated exposure to psychostimulants such as cocaine leads to sensitized response, long-term treatment with opiates (such as morphine) also has inverse consequences. The development of tolerance is a major problem in the clinical treatment of chronic pain and has been thought to play a central role in the opiate addiction and dependence as well. μ-opioid receptors become rapidly desensitized upon agonist stimulation and require higher and higher doses of the drug to produce similar cellular and behavioral responses. If the long-term administration of opiates to a dependent subject is then abruptly ceased, severe physical and psychological symptoms of withdrawal occur. Intuitively, it would make sense that the precipitation of the opiate withdrawal syndrome is caused by an altered homeostatic state where desensitized opioid receptors are no longer sufficiently stimulated in the absence of an exogenous agonist. However, studies in mice lacking β arrestin-2, a major component in the desensitization mechanisms of G protein-coupled receptors (see Figure 1), have shown that opiate tolerance and dependence are distinct processes (Bohn et al., 2000). In these animals, μ-opioid receptors will not become desensitized after morphine administration nor will the animals become behaviorally tolerant to the analgesic effects of morphine. Surprisingly, however, the opiate antagonist naloxone given to mutant mice treated chronically with morphine elicits withdrawal symptoms that are indistinguishable from those seen in wild-type animals. Thus, changes other than μ-opioid receptor desensitization, such as long-term changes in gene expression and cyclic AMP production, must be responsible for the withdrawal syndrome (Maldonado et al., 1996; Nestler, 2001a).

Phosphodiesterases break down the cyclic AMP generated by adenylyl cyclase, providing a way to terminate the intracellular signaling. Modulation of their activity could also lead to changed homeostatic equilibrium where the certain degree of receptor stimulation results in increased or decreased intracellular response. Phosphodiesterase 1B is a subtype that, in the brain, localizes to the regions innervated by the dopaminergic system, like striatum (Polli and Kincaid, 1994), and theoretically should work to dampen the dopaminergic signaling in the postsynaptic neuron. In line with this hypothesis, phosphodiesterase 1B knockout mice are hyperactive in a novel environment and show exaggerated locomotor responses to methamphetamine. Moreover, as expected, several protein kinase A substrates are hyperphosphorylated in response to D1 receptor agonists, including DARPP-32 at threonine-34 and GluR1-subunit of the AMPA receptor at serine-845 (Reed et al., 2002).

Somewhat surprising results were obtained with a mouse strain lacking the RIIβ-isoform of the cyclic AMP-dependent protein kinase (PKA) (Brandon et al., 1998). This isoform is enriched in striatum and is proposed to be one the most important PKA isoforms mediating the effects of dopamine. However, acute stimulatory effects of amphetamine and cocaine are intact and, even more strikingly, the mutant mice show enhanced sensitization to repeated amphetamine administration. In line with reduced PKA activity, however, c-Fos induction caused by amphetamine–presumably mediated by the phosphorylation of the transcription factor CREB (cyclic AMP responsive element binding protein) by PKA–is reduced in striatum. These results highlight the possibility that part of the same plasticity involved in the development of sensitization is also involved in tolerance against the drug's effects.

This same idea is suggested by results from mice either lacking (Hiroi et al., 1997) or overexpressing (Kelz et al., 1999) the transcription factor FosB. FosB, like c-Fos, is expressed, e.g., in response to CREB phosphorylation by PKA or Ca^{2+}/calmodulin-dependent kinases (CaM kinases). However, while sustained stimulation of this signaling pathway leads to a reduced induction of these early genes, the highly stable isoforms of FosB, known as ΔFosB, start to accumulate in the striatum. This accumulation of long-acting transcription factors is believed to mediate some of the long-term effects of psychostimulants and other psychotropic drugs (Nestler et al., 2001). Mice lacking the *fos*B gene are supersensitive to cocaine and show enhanced cocaine-conditioned place preference (Hiroi et al., 1997). Moreover, mice overexpressing ΔFosB in the nucleus accumbens also show heightened response to cocaine (Kelz et al., 1999). Taken together, these findings indicate that *fos*B gene products may have both suppressing and enhancing influences on the effects of psychostimulants.

One of the proteins downstream of ΔFosB that may participate in the negative feedback loop creating tolerance is cyclin-dependent kinase 5 (Cdk5). Cdk5 is upregulated in response to chronic cocaine exposure, as well as in ΔFosB overexpressing mice (Bibb et al., 2001). However, inhibiting the activity of Cdk5 by roscovitine leads to an enhanced sensitization to repeated cocaine administration. This effect appears to be mediated through changes in the phosphorylation state of phosphatase/kinase inhibitor DARPP-32. However, Cdk5 has numerous substrates and identifying them may provide clues for future targets of addiction therapy. Further substantiating the theoretical framework of intracellular equilibrium between tolerance and sensitization, mice overexpressing CREB in the nucleus accumbens display aversion to the effects of cocaine, whereas mice overexpressing a dominant-negative mutant of CREB are supersensitive to its rewarding effects (Carlezon et al., 1998). Eventually these mouse strains could be used as tools for forward genetics approaches, and the specific targets of the involved transcription factors can thus be identified.

Role of Glutamatergic Transmission in Addiction

In order to produce persistent behavioral patterns that may lead to a relapse even years after the previous drug exposure, drugs of abuse have to induce very long-lasting changes in the brain of an addicted subject. Because of the well-established role of the brain glutamate system in learning and memory in general, the same glutamatergic mechanisms have been postulated to underlie the plasticity that explains addiction-related behaviors (Berke and Hyman, 2000; Hyman and Malenka, 2001). Consistent with this hypothesis, drugs that block the NMDA-type glutamate receptors, such as MK-801, were originally reported to block the development of psychostimulant sensitization (Wolf, 1998; Vanderschuren and Kalivas, 2000) and were even proposed as a possible treatment for cocaine addiction. However, this was somewhat contradictory to the fact that NMDA antagonists like PCP and ketamine are abused themselves, and some research groups using different behavioral paradigms found that NMDA antagonists may even enhance sensitization to dopaminergic drugs (Tzschentke and Schmidt, 1998). Many of the inconsistencies in the field probably arose from different doses and treatment regimens, and it became apparent that genetic animal models could be invaluable for solving this issue.

NMDA receptor-deficient mice, expressing only 5% of the essential NR1 subunit of the NMDA receptor and 20%–25% of the NMDA receptor in different brain regions (Mohn et al., 1999), were recently tested for their responses to psychostimulants (Laakso et al., 2001, Soc. Neurosci. Abstr.). While the locomotor-stimulating effects of acute cocaine administration were almost abolished, repeated administration led to a fully sensitized locomotor response and full rewarding effect in a conditioned place-preference paradigm without any compensatory increase in NMDA receptor expression. This indicates that whatever plastic changes take place during the development of sensitization, they do not require intact NMDA receptor signaling. Surprisingly, however, responses to acute amphetamine administration at behavioral and gene expression levels were intact. Since cocaine and amphetamine increase extracellular dopamine levels through different mechanisms (inhibition of uptake and release from intracellular storages, respectively), it was first hypothesized that cocaine's lack of acute effects might be explained by the reduced ability of cocaine to elevate extracellular dopamine. However, striatal dopamine release induced by both acute cocaine and amphetamine administration was preserved in mutant animals. These results suggest an important role for corticostriatal glutamatergic neurotransmission in the acute effects of cocaine, whereas amphetamine's acute actions may be more directly mediated by dopamine. This pharmacodynamic difference (differential contribution of glutamatergic neurotransmission) between cocaine and amphetamine may also explain the dissociation between the acute behavioral efficacy of the two drugs and their potency to elevate striatal dopamine levels in wild-type animals: amphetamine increases extracellular dopamine levels two to five times more than cocaine yet is a less effective behavioral stimulant, indicating that additional (nondopaminergic) mechanisms must contribute to cocaine's acute effects (Kuczenski et al., 1991). From the viewpoint of addiction therapy, however, the results are somewhat disappointing and suggest that sensitization and reward may develop even in the absence of normal NMDA receptor signaling.

Metabotropic glutamate receptor 5 knockout mice were also found to be insensitive to the acute locomotor-stimulating effects of cocaine, but they did not self-administer it, even though they recognized food as an effective reinforcer (Chiamulera et al., 2001). Perhaps more importantly, however, the same group–inspired by the results in mutant mice–showed that the mGluR5 antagonist MPEP blocks cocaine self administration in wild-type animals. Of the many knockout mice tested to date in paradigms of drug addiction, the mGluR5 mutant mouse is the only line that fails to self-administer psychostimulants. Given the striking phenotype of these animals, it would be interesting to determine whether it

extends to other addictive drugs, such as morphine, nicotine, or ethanol.

Mice lacking the GluR1-subunit of AMPA-type glutamate receptors are slightly hyperactive in a novel environment, show supersensitive locomotor responses to morphine, develop less tolerance to its antinociceptive effects, and have milder naloxone-precipitated withdrawal symptoms after repeated morphine administration than wild-type mice (Vekovischeva et al., 2001). Furthermore, while context-independent sensitization (repeated drug injections not paired with the final test chamber) to the locomotor effects of morphine was missing in these mice, they developed normal context-dependent morphine sensitization and even enhanced context-dependent sensitization to the stimulating effects of amphetamine. While these results suggest a role for the AMPA receptors in both opioid and psychostimulant responses, more studies are needed to fully understand and reconcile their possibly opposing involvement in the actions of these different drugs of abuse.

Addiction studies in mice with genetically altered components of the brain glutamatergic system are still sparse. However, recent electrophysiological work in animals treated with cocaine and amphetamine has also demonstrated the crucial role of glutamatergic neurotransmission in the actions of these drugs. In VTA, even a short exposure to amphetamine blocks long-term depression (LTD) in glutamatergic synapses onto dopaminergic neurons (Jones et al., 2000), and single exposure to cocaine is able to induce long-term potentiation (LTP) of AMPA currents at the same synapses (Ungless et al., 2001). On the other hand, repeated cocaine exposure leading to sensitization induces long-lasting depression of AMPA receptor-mediated glutamatergic transmission at corticostriatal synapses in nucleus accumbens (Thomas et al., 2001; Beurrier and Malenka, 2002). The role of NMDA receptors in this plasticity, however, is not yet clear (Thomas et al., 2001; Beurrier and Malenka, 2002). Although this field is still young and evolving, the existing literature already suggests that similar glutamatergic mechanisms that underlie learning and memory in general may participate in the addiction process. More mutants with glutamatergic deficits will likely be screened in different drug-abuse paradigms in the future. This will hopefully also direct the attempts to develop new therapeutic tools for the treatment of addiction, for which drugs modulating excitatory neurotransmission hold a great promise.

Forward Genetics: A Phenotype Driven Approach

The theoretical framework that defines potential candidate genes inherently limits reverse genetic approaches. Gene targeting is best applied to answer the question of whether or not a gene is involved in an aspect of a phenotype. Forward genetics may provide a valuable complementary approach to find new genes or substrates that underlie addiction. Instead of mutating a candidate gene, forward genetics proceeds from a phenotype of altered drug response and searches for the genes responsible. In mice, such an approach could entail quantitative trait loci (QTL) mapping to define susceptibility loci for drug sensitivity, random mutagenesis and screening, or even microchip array analysis to find genes whose expression changes with drug administration. There are several genetic systems that can be used in a phenotype-to-genotype screen for genes related to addiction. There is evidence that, in addition to mice, zebrafish and *Drosophila* can be amenable to phenotypic screens for drugs of abuse. In this section, we will focus on the potential and progress that has been made in these three systems toward the identification of new genes that underlie addiction.

Drosophila as a Model System

As a classic system for forward mutagenesis screens, *Drosophila* has been useful, for example, in the identification and characterization of genes that are necessary for learning and memory. It may prove likewise useful for drug-abuse research (Wolf, 1999). Fruit flies respond to volatilized cocaine, nicotine, and ethanol with a range of behaviors that parallels mammalian behaviors. For cocaine and nicotine, there is a dose dependent shift from hyperactivity (lower doses), to stereotyped grooming, to paralysis (highest doses) (McClung and Hirsh, 1998; Bainton et al., 2000). Ethanol induces hyperactivity at low doses and sedation at higher doses (Bainton et al., 2000). Flies with altered drug response can be selected based on their impaired ability to perform climbing activities or negative geotaxis (fly up). McClung and Hirsh (1998) have also demonstrated that flies sensitize to cocaine, with increased responsiveness developing after repeated exposures for 2 days.

Drosophila screens have highlighted the importance of two biological pathways in the acute response to cocaine and ethanol. The first is the signal transduction pathway mediated by cyclic AMP. Impaired regulation of cAMP levels in a number of mutant fly lines confers enhanced sensitivity to ethanol (Moore et al., 1998). One of the first lines identified as such was *cheapdate*, which was selected from a forward screen for altered ethanol response. *Cheapdate* was subsequently identified as an additional hypomorphic allele of *amnesiac*, a memory mutant that is deficient for an adenylyl cyclase stimulating protein. Hypothesizing that reduced cAMP levels might be central to ethanol's actions, Moore et al. tested other known mutants with altered cAMP regulation, including *rutabaga* (calcium/calmodulin-sensitive AC), *dunce* (cAMP-phosphodiesterase), and *DCO* (catalytic subunit of cAMP-dependent protein kinase). *Rutabaga* and *DCO* mutations conferred enhanced ethanol sensitivity, while *dunce* mutant flies had normal responses to ethanol. Their results suggested that ethanol sensitivity is enhanced when cAMP signaling is impaired, but is not suppressed by excess cAMP accumulation (as is the case in *dunce* mutant flies). Within the context of genes of addiction, these studies are crucial for illuminating ethanol's still mysterious mechanism of action.

Cyclic AMP signaling has also been implicated in both the acute and sensitized responses to cocaine in *Drosophila*. Rather than taking a forward genetics approach, however, Park et al. (2000) studied the role of cAMP signaling in cocaine responsiveness by first identifying the *Drosophila* homolog of a gene and then characterizing a line with an insertional mutation in that gene. The *pka-RII* gene encodes a regulatory subunit of cAMP-

dependent protein kinase, and flies deficient in *pka-RII* display a number of related phenotypes including reduced sensitivity to both ethanol and cocaine and a lack of sensitization to cocaine.

These flies also displayed alterations in their circadian rhythm locomotor activity, highlighting the second biological pathway that has been implicated in *Drosophila* studies of addiction. Jay Hirsh and his colleagues have found that four other circadian rhythm genes are required for normal acute response and sensitization to cocaine (Andretic et al., 1999). The mutants *period*, *clock*, *cycle*, and *doubletime* fail to sensitize to cocaine, perhaps due to the fact that they also fail to induce tyrosine decarboxylase expression after a sensitization regimen. This thesis was further supported by findings that in the *Drosophila* mutant *inactive*, which harbors a mutant tyrosine decarboxylase gene, sensitization to cocaine is also abolished (McClung and Hirsh, 1999). This unexpected relationship between circadian rhythm, tyrosine metabolism, and cocaine responsiveness may illustrate best the potential for new theoretical constructs that could change our understanding of the process of addiction.

These studies in *Drosophila* have translated to the mouse. The two mouse homologs of *period*, *mPer1* and *mPer2*, have been disrupted by gene targeting (Zheng et al., 1999, 2001). Based on the correlation discovered in *Drosophila*, these mutants were tested for cocaine responsiveness, sensitization, and reward. Neither mutant showed alterations in acute cocaine response, but both showed striking and opposite alterations in sensitization and conditioned place preference. Mice lacking *mPer1* fail to become sensitized or display conditioned place preference, whereas mice lacking *mPer2* display enhanced sensitization and reward (Abarca et al., 2002).

Forward Genetics in the Mouse

Some of the first studies of the genetics of addiction using mouse models capitalized on the identification of inbred strains displaying differential alcohol preference (McClearn and Rodgers, 1959). This observation has been the starting point for a considerable amount of research to identify quantitative trait loci (QTL) for various aspects of alcohol and drug addiction. A QTL is the chromosomal location of a gene that contributes to the expression of a phenotype, such as enhanced ethanol sensitivity. QTL mapping begins with two inbred strains that differ substantially in some aspect of drug response. The recombinant inbred lines from these parental strains display a continuum of phenotypes that can extend beyond those of the original strains. A QTL is then identified when a phenotype shows strong genetic linkage to a chromosomal region in several recombinant inbred lines. With improved interstrain polymorphism markers and the completion of the murine genome sequence, QTL mapping is poised to reveal the identity of the alleles that confer altered responses to ethanol (see Crabbe and Phillips, 1998, for review), morphine (Belknap and Crabbe, 1992), cocaine (Tolliver et al., 1994; Miner and Marley, 1995; Phillips et al., 1998), amphetamine, and phencyclidine (Alexander et al., 1996).

The QTL method of genetic analysis tells us only what naturally occurring alleles can influence drug response and addiction. However, the same genetic approach could theoretically be used with chemically induced mutations to cover every allele in the genome. Ethylnitrosourea (ENU) has been used for many years to generate point mutations in the mouse germline that result in null, hypomorphic, and hypermorphic alleles (Justice et al., 1999). In contrast to insertional mutagenesis that can generate a single mutation per mouse, ENU has a mutation rate that can effectively saturate the genome. Specific locus tests, which detect the frequency of null mutations at seven loci in the mouse, suggest that ENU has a forward mutation rate of as high as one null mutation for every 650 genes (Hitotsumachi et al., 1985). Given the ability to theoretically mutate one allele of every gene in a population of as little as 300–900 mice, it is not inconceivable to propose a genome-wide screen for mutations that affect some aspect of drug-taking behavior. One principle advantage of such a screen is that like QTL mapping, it is an unbiased approach that may uncover novel genes whose function is not known or is not currently considered in drug abuse. However, this is not to say that it is without limitations; such endeavors require significant numbers of mice to map and clone a gene (Vitaterna et al., 1994; King et al., 1997). Furthermore, if a screen focuses on one aspect of drug-taking behavior, such as acute response to a drug, it may miss those genes that influence another aspect, such as relapse potential. Therefore, the power of this forward genetic approach is also dependent on the quality of the phenotypic screen. For practical reasons, screens for complex behaviors (such as relapse or self administration) are usually less realistic with such a large numbers of animals.

Gene Expression Profiling

If forward genetics is defined as a method that starts with a phenotype and ends with a gene, another experimental method that should be discussed in this context is expression analysis using differential display and gene chip microarrays. Using these technologies, changes in the gene expression profile in relevant brain regions (e.g., nucleus accumbens) have been assessed following chronic cocaine treatment in mice. The first studies using differential display have highlighted the role of some unexpected genes. A putative transcriptional regulator (NAC-1), a G protein β subunit (rGβ1), and a transcript encoding a new peptide transmitter or signaling molecule (CART) have all been isolated from the brains of rats treated with cocaine or amphetamine (Cha et al., 1997; Wang et al., 1997; Douglass and Daoud, 1996). More recently, investigators have used gene chip microarrays to detect changes in gene expression. Evidence from this line of research has implicated NF-κB (Ang et al., 2001) and cyclin-dependent kinase 5 (Bibb et al., 2001) in signal transduction pathways that modulate cocaine's behavioral effects and contribute to long-term neuronal changes associated with addiction. These results are undoubtedly the first of many. While research may focus initially on those genes with known function, it should also assist in the functional annotation of the novel genes whose expression changes in response to drugs of abuse.

Zebrafish as a Potential Model System

Zebrafish, as a relatively new genetic system, has obviously not been used extensively in the field of drug addiction. However, there are preliminary reports suggesting that they can be used for screens of the acute response to ethanol (Gerlai et al., 2000) and cocaine (Darland and Dowling, 2001). Gerlai et al. describe a series of behaviors that are altered by ethanol exposure in a dose-dependent manner, including activity, social behavior, and light-dark/top-bottom preferences. Although their report did not extend to screening mutagenized fish, it established the feasibility of future screens to assess acute response to ethanol. In a different study, Darland and Dowling performed a chemical mutagenesis screen to study cocaine responsiveness; conditioned place preference was measured by placing a cocaine-saturated wick in one side of a tank divided by a perforated wall. While most fish display preference for the cocaine-treated side of the tank, three mutant fish lines were identified that showed no preference: *dumbfish* (*dum*), *jumpy* (*jpy*), and *goody-two-shoes* (*gts*). The spatial learning and memory of these fish were tested in a simple T maze assay, with *dum* showing impaired performance but *jpy* and *gts* showing normal performance. These studies show promise for uncovering genes related to addiction that may be evolutionarily conserved among vertebrates.

Challenges and Future Directions for the Genetics of Addiction

The collection of transgenic mice that have an altered response to at least one drug of abuse has grown quite large. The question remains as to whether all of the genes thus implicated are in fact responsible for the neuronal plasticity that leads to the initiation and maintenance of the addictive state. One way to address this question is to identify those mice that display altered response to several classes of addictive drugs. This subset of mice would presumably have mutations in the genes that are key players in determining the addictive process.

Another way to determine which genes are most crucial in addiction is to characterize these mice with more sophisticated behavioral paradigms. Most studies of genetically altered mice have focused on the early behavioral manifestations of drug response: acute response, sensitized response, and conditioned place preference. The next step in the characterization of these animals should be to examine some of the more complex behaviors relevant to addiction, such as self-administration and relapse. This testing will be necessary to distinguish the genes that mediate acute response from those that mediate neuronal plasticity underlying addiction.

If one were to characterize a mutant mouse line with all of the available behavioral tests, what would the ideal mouse look like? What is the best model for understanding the process of addiction? Any mouse that fails to establish a normal response to a drug of abuse is informative. It is relatively easy to identify mutants that fail to respond acutely or are supersensitive to a drug. It is also feasible to identify mutants that do not prefer drug or self-administer drug. All of these mice would help pinpoint those genes that are involved in the establishment of addiction. However, the challenge will be to identify those genes that are involved in the extinction of addiction. In order to find therapeutic targets for human drug addicts, the ideal mutant should develop an addiction but then be more resilient to relapse.

Clearly, the benefit of these studies ought to be the eventual application to human drug addiction. One application may be the identification of susceptibility loci in humans. Genes central to addiction in animal models may have differing alleles in humans, and allelic differences may explain in part why some people are more susceptible to addiction than others. The earliest and best-known example of this is the identification of the *Taq*1 A1 allele of the human D2 dopamine receptor gene as a risk factor for alcoholism (Blum et al., 1990). However, it has proven extremely difficult to define the genes responsible for polygenic disorders, which most neuropsychiatric disorders (like addiction) undoubtedly are. When one gene contributes only 1%–5% to the total risk, half of which is environmental instead of genetic, one quickly realizes the difficulty of the task at hand (Comings, 1998; Duaux et al., 2000; Nestler, 2001b). Still, at some future date, perhaps people with an identified susceptibility to addiction would be aware of their genetic predisposition and would be less likely to engage in experimental drug use. But more desirable than this scenario would be the identification of genes that prevent relapse in humans. It is only after an addiction is established and reward pathways are altered when patients do seek to cease drug use. Ideally, genetic studies in animals should help identify therapeutic targets to assist in relapse prevention. While it will be challenging to determine which genes or changes are uniquely responsible for addiction, understanding the process in greater detail should lead to novel approaches to develop therapeutic or preventative interventions.

Acknowledgments

Work from the authors' laboratory is supported in part by grants from the NIH: DA-13511, MH-40159, NS-19576, and a grant to the Consortium for Mouse Genetics of Addiction from the Zaffaroni Foundation. M.G.C. is an investigator of the Howard Hughes Medical Institute.

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